Regeneration of transgenic loblolly pine expressing genes for salt tolerance

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Abstract: Salinity stress is one of the most serious factors limiting the distribution and productivity of crops and forest trees. The detrimental effects of salt on plants are a consequence of both a water deficit resulting in osmotic stress and the effects of excess sodium ions on critical biochemical process. A novel approach to improve salt tolerance has been established by using the technology of plant genetic transformation and using loblolly pine (*Pinus taeda* L.) as a model plant. Mature zygotic embryos of loblolly pine were infected with *Agrobacterium tumefaciens* strain LBA 4404 harbouring the plasmid pBIGM which carrying the mannitol-1-phosphate dehydrogenase (Mt1D) and glucitol-6-phosphate dehydrogenase (GutD). Organogenic transgenic calli and transgenic regenerated plantlets were produced on selection medium containing 15mg/L kanamycin and confirmed by Southern blot analysis of genomic DNA. Salt tolerance assays demonstrated that the salt tolerance of transgenic calli and regenerated plantlets were increased. These results suggested that an efficient *Agrobacterium tumefaciens*-mediated transformation protocol for stable integration of foreign genes into loblolly pine has been developed and this could be useful for the future studies on engineering breeding of conifers.

Keywords: *Pinus taeda* L.; *Agrobacterium tumefaciens*; Salt tolerance; Genetic engineering

Abbreviations: BA: benzyladenine; 2,4-D: 2,4-dichlorophenoxyacetic acid; GutD: glucitol-6-phosphate dehydrogenase; IBA: indole-3-butyric acid; Mt1D: mannitol-1-phosphate dehydrogenase; nptII: neomycin phosphotransferase II gene

CLC Number: Q55; S791.255.04 **Document code**: A Article ID: 1007-662X(2002)01-0001-06

Introduction

Accumulation of salts in irrigated soil is primary factors depressing forest production, because most of the forest trees are almost universally non-halophytic (Serrano 1996; Volkmar *et al.* 1998). Organisms that thrive in hypersaline environments possess specific mechanisms to adjust their internal osmotic status by the ability to accumulate low molecular weight organic compatible solutes such as sugars, some amino acids and quaternary ammonium compounds, which are believed to be essential for adaptability of plant cells to high salinity (Yancey *et al.* 1982). Expression of compatible solutes and heterologous sodium efflux transporters may be a useful approach to improve the salt tolerance of forest trees. Indeed, the increase of salt tolerance or water stress tolerance of photosynthetic organisms transformed with genes for synthesis of compatible solutes was demonstrated (Nomura *et al.* 1995; Takabe *et al.* 1998). In addition to these toxic effects, salt stress also causes the induction of oxidative stress (Burdon *et al.* 1996). Thus, the enhancement of enzyme activity involved in active oxygen scavenging systems may be a potent strategy to increase salt tolerance (McKersie *et al.* 1996).

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Received date: 2001-08-08

Responsible editor: Chai Ruihai

Although genetic transformation of conifers has made great progress recently (Klimaszewska *et al.* 1997; Walter *et al.* 1999), it lagged behind the advances made in angiosperm forest trees and herbaceous crops due both to economics and the recalcitrant nature of woody perennials to in vitro manipulation. Transient expression from microprojectile and *Agrobacterium tumefaciens*-mediated gene transfer has been reported for *Pinus radiata* (Bergmann and Stomp 1992), *Pinus pinea* (Humara *et al.* 1999), *Pinus halepensis* (Tzfira *et al.* 1996), and larch (Diner and Karnosky 1987). To date, stable transformation of conifers using biolistics and *Agrobacterium tumefaciens*-mediated has been achieved for *Larix decidua* (Huang *et al.* 1991), *Picea abies* (Walter *et al.* 1999), *Larix laricina* (Klimaszewska *et al.* 1997), *Picea glauca* (Ellis *et al.* 1993), *Pinus radiata* (Walter *et al.* 1998), larch (Shin *et al.* 1994), and hybrid larch (Levee *et al.* 1997). Loblolly pine is an important forest species in the tropical and subtropical region. Sederoff *et al.* (1986) first reported the gene transfer by *Agrobacterium tumefaciens* in loblolly pine. Stomp *et al.* (1991) succeeded in transient expression from microprojectile-mediated DNA transfer in *Pinus taeda*. Yet there is no report on the transfer of mannitol-1-phosphate dehydrogenase (Mt1D) gene and glucitol-6-phosphate dehydrogenase (GutD) gene by *Agrobacterium tumefaciens*-mediated transformation into loblolly pine. Therefore, we investigated whether the mannitol-1-phosphate dehydrogenase (Mt1D) gene and glucitol-6-phosphate dehydrogenase (GutD) gene can be transferred into loblolly pine plants that will confer salt resistance. Here, we report that

we have produced transgenic loblolly pine plants that have increased their salt tolerance. This investigation could be useful for the future studies on genetic engineering breeding of conifers, as well as other forest trees.

Materials and methods

Plant materials

Mature seeds of loblolly pine (genotypes Hb, Ma, and Mc) were collected from Shaoyang Loblolly Pine Seed Orchard, Hunan Province, China in October 1996. Mature seeds of loblolly pine genotypes J-56 and S-1003 were kindly provided by Professor ZHONG Weihua. All seeds were stored in plastic bags at 4oC before they were used to tissue culture. Seeds were disinfected by immersion in 70% w/w ethanol alcohol for 30 s and in 0.1% mercuric chloride for 10-15 min, followed by four to five rinses in sterile distilled water. Mature zygotic embryos were aseptically removed from the megagametophytes and placed horizontally on a solified callus induction medium in flasks or Petri dishes. Mature zygotic embryo explants were used to transformation experiments after cultured on pretreatment medium consisted of TE medium (Tang *et al.* 1998) supplemented with 8 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 4 mg/L benzyladenine (BA), and 4 mg/L kinetin for 1-3 weeks.

Agrobacterium strain and plasmid construction

Agrobacterium tumefaciens strain LBA4404 containing the binary plasmid pBIGM (Li *et al.* 1995) which carrying the mannitol-1-phosphate dehydrogenase (Mt1D) gene, glucitol-6-phosphate dehydrogenase (GutD) gene, and neomycin phosphotransferase (nptII) gene which confers kanamycin resistance was used in the transformation experiments. The mannitol-1-phosphate dehydrogenase (Mt1D) gene and glucitol-6-phosphate dehydrogenase (GutD) gene are under the control of the double cauliflower mosaic virus 35S promoter and the terminator from nopaline synthase (nos) gene. The neomycin phosphotransferase (nptII) gene is under the control of the cauliflower mosaic virus 35S promoter and the terminator from nopaline synthase (nos) gene (Li *et al.* 1995). *Agrobacterium tumefaciens* was grown overnight at 28°C in liquid YEP medium (Sambrook *et al.* 1989) supplemented with 50 mg/L kanamycin. The overnight culture was used for transformation of mature zygotic embryos. Concentration of bacterium was determined in MILTON ROT spectronic 1201 at 600 nm.

Co-cultivation, selection, and regeneration.

After mature zygotic embryos were infected with *Agrobacterium tumefaciens* LBA 4404 cultures (OD600: 0.5-1.0) for 15-25 min, co-cultivation was conducted at 25°C for 3-5 days in the dark on callus induction medium consisted of TE medium (Tang *et al.* 1998) supplemented with 8 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 4 mg/L benzyladenine (BA), 4 mg/L kinetin, and 50 (M coniferyl alcohol (Stachel *et al.* 1985). Co-cultivated mature zygotic embryos were washed 4-5 times in sterile distilled water for reducing the *Agrobacterium* contamination, and were placed on sterile filter paper to remove excess liquid and transferred onto callus induction medium containing 500 mg/L carbenicillin (for killing *Agrobacterium tumefaciens* LBA4404) for one week. Then co-cultivated mature zygotic embryos were transferred onto callus induction medium supplemented with 500 mg/L carbenicillin and 15 mg/L kanamycin (for killing non-transformed cells and tissues). After three weeks, mature zygotic embryos were transferred to fresh callus induction medium. After organogenic calli were initiated from transformed mature zygotic embryos cultured on induction medium with 15 mg/L kanamycin, Plant regeneration was carried out on differentiation supplemented with selection pressure according to a procedure previously described (Tang *et al.* 1998). Differentiation was evaluated by the percentage of calli forming transgenic adventitious shoots on medium for shoot regeneration for 9 weeks. Rooting was evaluated by the percentage of transgenic shoots forming roots on medium for root regeneration for 6 weeks. All media were supplemented with 3 % sucrose and 0.3% Phytagel (Sigma). Media were adjusted to pH 5.8 prior to autoclaving 20 min at 121 °C. All cultures were cultured at 25 °C culture room. Adventitious shoot induction was conducted in the dark and adventitious shoot differentiation and proliferation and rooting were conducted at 25 °C under a 16-h photoperiod with cool fluorescent light (100 $\mu mol \cdot m^{-2} \cdot s^{-1}$). After acclimatization of regenerated plantlets was conducted by decreasing relative humidity to ambient condition over a period of one week, plantlets were established in soil. Data were analyzed by the Analysis of Variance (ANOVA).

Southern blots

Genomic DNA from 4 months old non-transformed control and putative transgenic plants according to the methods of Wagner *et al.* (1987). Twenty micrograms of genomic DNA was digested overnight with the restriction enzyme EcoRI and Hind III (Boehringer Mannheim) at 37°C, according to the manufacturer's instructions and separated on a 0.8% agarose gel. The DNA was transferred to Pall Biodyne A membranes (Pall, Muttenz, Switzerland) and blots were hybridized with Mt1D probe which was labeled with 32P-dCTP (Ready to Go Labeling Beads (Pharmacia)), according to standard protocols (Sambrook *et al.* 1989). Membranes were washed twice in 2×SSC, 0.1% SDS at 65°C for 5 min each, once in 0.5 × SSC, 0.1 % SDS at 62°C for 15 min, and once in 0.1×SSC, 0.1% SDS at room temperature for 30 min, and exposed to Kodak X-Omat-AR films at –80 °C for three days.

Scanning electron microscopy

Samples were prepared for scanning electron microscopy at different stages of culture. Tissues were fixed overnight in 4% glutaraldehyde and 100 mM phosphate buffer (pH

7.0), washed one time in 100 mM phosphate buffer (pH 7.0) for 30 min, followed by dehydration in successive ethanol solutions of 85%, 95%, and 100%, each repeated twice for 5 min. Specimens were dried in a critical-point-drier with CO_2 for 2 h, mounted on Cu stubs and gold-coated. The samples were examined and photographed in HITACHI S-800 scanning electron microscope.

Salt tolerance assay

Salt tolerance of transgenic loblolly pine plants was evaluated by the method of Hoshida *et al.* (2000) with some modification. For salt stress of transgenic calli, 9-week old transgenic calli were transferred from proliferation medium (Tang *et al.* 1998) to 5-fold-diluted TE basal medium (Tang *et al.* 1998) containing 0.5% and 0.7% NaCl, respectively. Survival rate of transgenic calli was determined in the 6th week of salt stress. For salt stress of transgenic plants, transgenic plants 4-5 cm in height were transferred from differentiation medium (Tang *et al.* 1998) to 5-fold-diluted TE basal medium containing 0.5% and 0.7% NaCl, respectively. Survival rate of transgenic plants was determined in the 6th week of salt stress. Non-transformed calli and regenerated plants were used as the control for the same salt stress. Data were analyzed by the Analysis of Variance (ANOVA).

Results and discussion

Transformation and transgenic plant regeneration

Before the transformation experiments, the optimal concentration of kanamycin for selecting transformed cells and tissues was determined by culturing mature zygotic embryos on callus induction medium containing different concentration of kanamycin. Experimental results demonstrated that 15mg/L kanamycin was the best one to identify transformed cells and tissues. Two to three weeks after infected mature zygotic embryos were transferred onto callus induction medium supplemented with 15mg/l kanamycin, mature zygotic embryos began to form calli. The frequency of transgenic calli increased during 3-8 weeks on fresh callus induction medium supplemented 15mg/l kanamycin. The highest frequency of transgenic callus formation was obtained from genotype Mc. Callus was formed on cotyledons, hypocotyl, and radicles of embryos two to three weeks after infection (Fig. 1a). Proliferation of transgenic calli (Fig. 1b) was achieved by sub-cultured kanamycin resistant calli on fresh callus induction medium with kanamycin.

Putative transgenic adventitious shoots were formed on the surface of kanamycin resistant calli (Fig. 1c) weeks after kanamycin-resistant calli were transferred to differentiation medium. The frequency of adventitious bud formation was 6.5%-14.9% on the differentiation medium supplemented with BA and indole-3-butyric acid (IBA) (Fig. 2). Proliferation of transgenic shoots was finished by sub-culturing organogenic calli in fresh differentiation medium (Fig. 1e). Cluster of transgenic shoots was formed on fresh differentiation medium (Fig. 1f). Both rooting of adventitious buds and acclimatization of regenerated plantlets (Fig. 1g) were carried out according to a procedure previously described (Tang *et al.* 1998). Rooting frequency 35.8%-74.9% was observed (Fig. 3) and both growth and phenotype of regenerated plantlets appeared similar to the untreated control. Regenerated plantlets from transgenic organogenic calli of loblolly pine were transferred from culture flasks into a perlite:peatmoss:vermiculite (1:1:1 v/v/v) soil mixture, and acclimatized plantlets were successfully established in the field (Fig. 1h). There are no obvious difference in induction frequency of transgenic shoots and rooting frequency of transgenic shoots between transgenic materials and non-transformed control (Fig. 2, 3).

Confirmation of T-DNA integration

Trangenic plantlets from independent transformation events were analyzed by Southern hybridization. Genomic DNA was isolated from control and putative transgenic plants according to the methods of Wagner *et al.* (1987). Twenty micrograms of DNA was digested overnight with the restriction enzyme EcoRI and Hind III (Boehringer Mannheim) at 37 °C and was used in Southern hybridisation experoments. No bands were detected in non-transformed control plants, whereas bands were observed in transgenic plants (Fig. 1d). These results confirm the presence of foreign genes integrated into the *Pinus taeda* genome. The Southern results of regenerated transgenic plants demonstrated that one band which represented junctions between T-DNA and adjacent plant DNA. These findings show that these are transformants in which the insert DNA containing Mt1D and GutD genes has integrated at one site in the plant genome. The integration of foreign genes in regenerated transgenic plants was also confirmed in *Pinus radiata* via particle bombardment (Walter *et al.* 1998).

Salt tolerance in transgenic loblolly pine plants

To examine whether the transgenic calli and transgenic plants derived from different genotypes of loblolly pine conferred resistance to salt stress, 9-week old transgenic calli and transgenic plants 4-5 cm in height were transferred to 5-fold-diluted TE medium containing 0.5% and 0.7% NaCl, respectively. The effects of NaCl on the survival rate of transgenic calli and transgenic plants were examined. The control calli and regenerated plants were treated with NaCl in the same experimental conditions and we have detected the much low survival rate for non-transformed calli (Table 1) and non-transformed regenerated plants (Table 2) after 6 weeks. However, the transgenic calli (Table 1) and transgenic plants (Table 2) remained at high survival rate at 0.5% and 0.7% NaCl, respectively. Salt tolerance of transgenic calli and transgenic plants differ among different genotypes of loblolly pine.

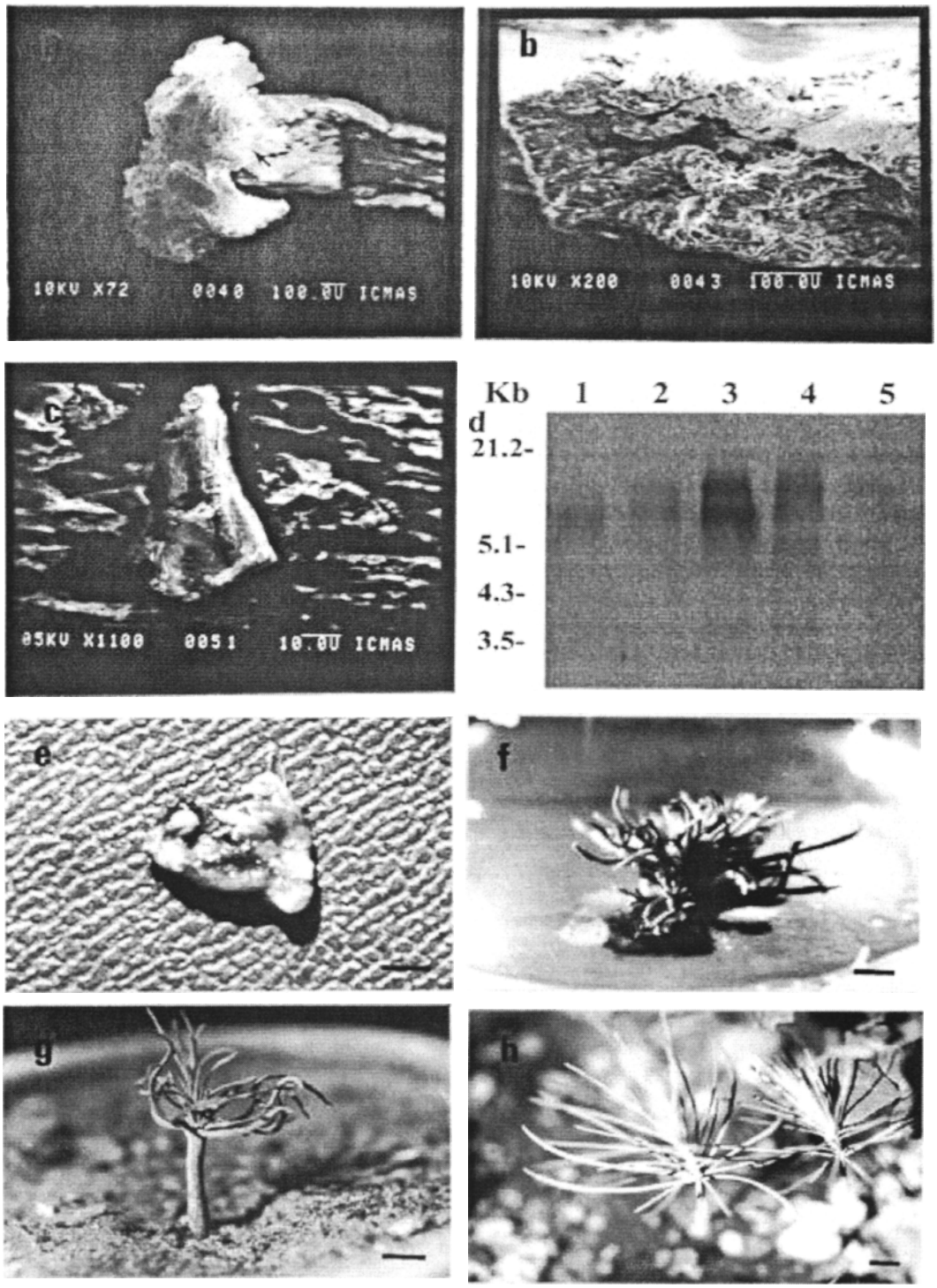

Fig. 1 *Agrobacterium tumefaciens*-mediated transformation and molecular analysis of transgenic plant in loblolly pine.

(a) Kanamycin-resistant callus derived from infected mature zygotic embryos; (b) Proliferation of kanamycin-resistant callus derived from cotyledon; (c) Kanamycin-resistant adventitious shoot; (d) Southern blot analysis of transgenic loblolly pine plants. DNA was digested with the restriction enzyme EcoRI and Hind III (Boehringer Mannheim) overnight at 37 °C, was hybridized (at 65 °C) with the Mt1D probe (BamHI-SacI fragment of B.t. gene), which was labeled with 32P-dCTP (Ready to Go Labeling Beads (Pharmacia), lane 1-3 DNA from transgenic plants of genotype Hb, Ma, and Mc, respectively (20 μg), lane 4 plasmid DNA of pBIGM (5pg); lane 5 DNA from non-transformed plant of genotype E-311 (20 μg); (e) Proliferation of transgenic shoots (Bar = 1 cm); (f) Cluster of transgenic shoots (Bar = 1.2 cm); (g) Acclimatization of transgenic regenerated plantlets in greenhouse (Bar = 1.5 cm); (h) Transgenic regenerated plantlets established in soil (bar = 1.5 cm).

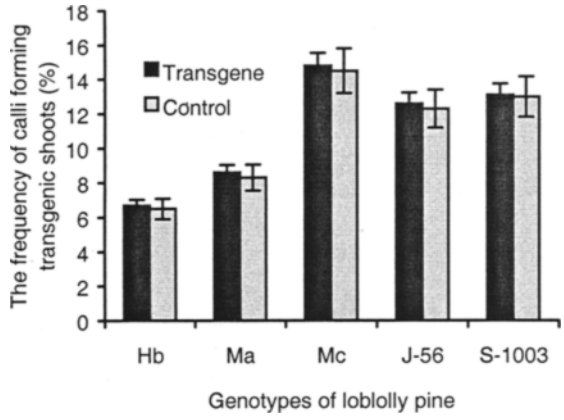

Fig. 2 Influence of genotypes on the differentiation frequency of transgenic calli in loblolly pine.

(Experiments were repeated three times and each replicate consisted of 30 pieces of transgenic calli 0.5 cm × 0.5 cm in size. The frequency of calli forming transgenic shoots was determined in the 9th week of culture. Values represent the means ± S.D.)

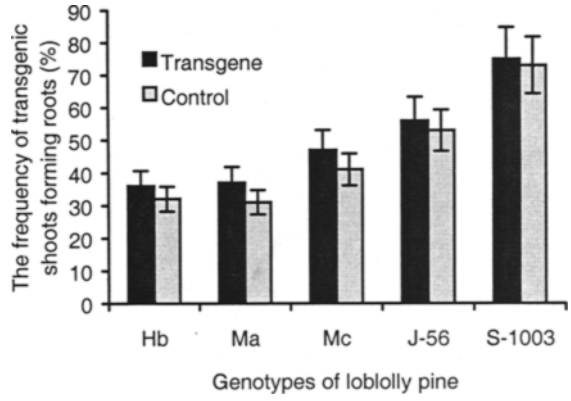

Fig. 3 Influence of genotypes on the rooting frequency of transgenic shoots in loblolly pine.

(Experiments were repeated three times and each replicate consisted of 30 transgenic shoots 4-5 cm in height. The frequency of transgenic shoots forming roots was determined in the 6th week of culture. Values represent the means ± S.D.)

High survival rate of transgenic calli was obtained from genotype Mc (19.1% at 0.5% NaCl and 14.8% at 0.7% NaCl, respectively). High survival rate of transgenic plants was obtained from genotype S-1003 (19.5% at 0.5% NaCl and 14.6% at 0.7% NaCl, respectively). Compared to the control, the survival rate of transgenic calli at 0.5% and 0.7% NaCl increased for all genotypes tested with the highest increase in genotype Mc (Table 1). The survival rate of transgenic plants at 0.5% and 0.7% NaCl increased for all genotypes tested with the highest increase in genotype S-1003 (Table 2). The results presented here clearly show that the Mt1D and GutD transgenic calli and transgenic regenerated plants of loblolly pine can increase their salt adaptability and improve their salt tolerance. To our knowledge, this is the first investigation that demonstrated that salt tolerance may be improved by transfer Mt1D and GutD genes into genome in conifers.

Table 1. Salt tolerance analyses of transgenic calli from different genotypes of loblolly pine

Genotypes	Survival rate of transgenic calli (%)			
	0.5% NaCl		0.7% NaCl	
	Transgenic calli	Control	Transgenic calli	Control
Hb	15.6±4.5	7.2±1.1	12.1±4.3	2.7±0.8
Ma	16.9±3.7	6.7±1.3	13.7±3.6	2.7±1.1
Mc	19.1±4.3	5.1±1.2	14.8±3.7	3.8±1.3
J-56	17.3±3.8	6.8±1.8	11.6±3.9	2.6±1.4
S-1003	17.5±5.3	7.3±1.5	12.3±3.4	3.2±1.1

Experiments were repeated three times and each replicate consisted of 30 pieces of transgenic calli 0.5 cm × 0.5 cm in size. Survival rate of transgenic calli was determined in the 6th week of salt stress. Values represent the means ± S.D.

Table 2. Salt tolerance analyses of transgenic regenerated plants from different genotypes of loblolly pine

Genotypes	Survival rate of transgenic plants (%)			
	0.5% NaCl		0.7% NaCl	
	Transgenic plants	Control	Transgenic plants	Control
Hb	15.7±3.5	6.5±1.2	12.3±1.3	2.8±1.8
Ma	16.3±3.7	5.7±2.3	11.7±1.8	2.7±1.5
Mc	18.1±2.3	5.1±1.5	12.8±1.9	3.9±1.3
J-56	16.9±4.7	7.2±1.8	11.6±2.9	2.4±1.8
S-1003	19.5±5.2	6.3±1.5	14.6±1.4	3.9±1.7

Experiments were repeated three times and each replicate consisted of 30 transgenic plants 4-5 cm in height. Survival rate of transgenic plants was determined in the 6th week of salt stress. Values represent the means ± S.D.

Acknowledgements

I wish to thank Professor C. Koncz, Professor R. Martienssen, Professor X.X. Peng, and Professor Ronald Sederoff for encouragement and support.

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